Conjugates for Influencing Interactions Between Proteins

The present invention relates to a conjugate which is suited to influence interactions between proteins, a DNA encoding such a conjugate and the use of the conjugate.

Many processes occurring in an organism are based on interactions between proteins. Examples of such interactions are found in receptors and the ligand binding thereto. However, the interactions between proteins are often unbalanced. This may be due to the fact that individual proteins involved in the interactions are modified, so that their affinity for other proteins which are also involved, is changed. Individual proteins involved in the interactions may also be lacking. This is found e.g. in the case of cells which do not respond to interleukin-6 (IL-6). Such cells have an incomplete interleukin-6 receptor, i.e. this receptor merely comprises the intracellular signal-triggering subunit gp130 but not the extracellular, IL-6-binding subunit (IL-6R).

Many attempts have been made to remedy unbalanced interactions between proteins. For example, this is tried in the case of an incomplete inleukin-6 receptor by administration of IL-6 (50 ng/ml) and soluble IL-6R (sIL-6R) (1280 ng/ml). However, the provision of sIL-6R is expensive and time-consuming, since sIL-6R will only be biologically active if it originates from eukaryotic cells, and the yields therefrom range from 1 to 6 mg sIL-6R/l. Thus, said administration is no suitable means to lastingly remedy the unbalanced interactions in the case of an incomplete interleukin-6 receptor.

Therefore, it is the object of the present invention to provide a product by which unbalanced interactions between proteins can be remedied, particularly in the case of an incomplete interleukin-6 receptor.

According to the invention this is achieved by the subject matters defined in the claims.

Thus, the subject matter of the present invention relates to a conjugate comprising two polypeptides with a mutual affinity, the polypeptides being linked with each other via a linker.

The expression "polypeptides with a mutual affinity" relates to polypeptides of any kind, origin and length, which have an affinity for each other. Two such polypeptides are present in a conjugate according to the invention. One of these polypeptides may be a receptor and the other may be a ligand binding to the receptor. The receptor may be present in the form of its subunit and the functional part thereof, respectively, which are capable to bind the ligand. Likewise, the ligand may be present in the form of its subunit and the functional part thereof, respectively, which are capable to bind the receptor. The receptor is preferably a cytokine receptor, particularly a receptor for lymphokines, monokines, interferons, colony stimulating factors or interleukins. It is especially preferred for the receptor to be an interleukin-6 receptor or a CNTF receptor. The same applies correspondingly to the ligand. It is preferably a cytokine, particularly a lymphokine, monokine, interferon, colony stimulating factor or interleukin. It is especially preferred for the ligand to be a member of the interleukin-6 family, particularly IL-6, IL-11, CNTF, OSM, LIF or CT-1. The receptor and the ligand may comprise wild-type sequences or sequences differing therefrom by one or several nucleotides. As a result, the receptor and the ligand may have improved and/or new properties. For example, improved properties may be represented by the fact that the bond between receptor and ligand is improved. For example, new properties may be represented by the fact that the ligand shows a behavior modified with respect to proteins with which it reacts after binding to the receptor. For example, IL-6 may be modified to the effect that it binds more strongly to the IL-6 receptor, but can no longer

activate the protein gp130. In such a case, IL-6 comprises preferably the sequence of fig. 3 or fragments thereof. The above statements made on a modification of the wild-type sequence of a receptor and a ligand, respectively, apply correspondingly to the other subunits and functional parts thereof, which contribute to a mutual bond.

The expression "linker" refers to linkers of any kind, which are suited to bind polypeptides. Examples of such linkers are bifunctional, chemical cross-linkers, e.g. DPDPB. Moreover, the linker may be a disulfide bridge formed by both polypeptides. In addition, the linker may be a polypeptide.

In a preferred embodiment, an above conjugate is a fusion polypeptide. It may contain the two polypeptides which have a mutual affinity and are fused to each other, and the linker may represent a disulfide bridge formed by the two polypeptides. The linker is preferably a polypeptide which binds the two other polypeptides with each other. Examples of the latter fusion polypeptide are indicated in figs. 1 and 2. These fusion polypeptides comprise a human sIL-6R polypeptide, i.e. the extracellular subunit of an interleukin-6 receptor, and a human IL-6 polypeptide, the polypeptides being linked with each other via differing polypeptide linkers. These fusion polypeptides are referred to as H-IL-6. A variation of H-IL-6 which only contains the amino acids Pro 114 to Ala 323 of the sIL-6R polypeptide, is also provided. Furthermore, a variation of H-IL-6 is provided which comprises amino acids 113 to 323 of the sIL-6R polypeptide and amino acids 29 to 212 of the IL-6 polypeptide. In addition, a fusion polypeptide H-IL-6 is provided whose IL-6 polypeptide comprises the sequence of fig. 3. The sIL-6R polypeptide of this fusion polypeptide comprises a complete sequence and the sequence between amino acids 113 (114) to 323 of an sIL-6R polypeptide, respectively. Besides, a fusion polypeptide is provided which comprises the extracellular subunit of a human CNTF

receptor and human CNTF, both polypeptides being linked with each other via a polypeptide linker.

A further subject matter of the present invention relates to a DNA coding for an above fusion polypeptide. The DNA codes preferably for a fusion polypeptide in which both polypeptides with a mutual affinity are linked with each other via a polypeptide linker. An example of the latter DNA is indicated in fig. 1. This DNA was deposited with the DSM (*Deutsche Sammlung von Mikroorganismen und Zellkulturen* [German-type collection of micro organisms and cell cultures]) as CDM8-H-IL-6 under DSM 10549 on February 27, 1996.

A DNA according to the invention can be present in a vector and expression vector, respectively. A person skilled in the art is familiar with examples thereof. In the case of an expression vector for E. coli these are e.g. pGEMEX, pUC derivatives, pGEX-2T, pET3b and pQE-8. For the expression in yeast, e.g. pY100, Ycpad1 and vectors for Pichia pastoris have to be mentioned, the latter being preferred, while for the expression in animal cells, which may be present within an organism or outside thereof, e.g. pKCR, pEFBOS, pCEV4 and pCDM8 have to be indicated, the latter being preferred. The baculovirus expression vector pAcSGHisNT-A is especially suitable for the expression in insect cells. The person skilled in the art will take into consideration that for the expression of a DNA according to the invention, which contains sIL-6R sequences, it is advisable to use vectors which enable an expression in eukaryotic cells.

However, the person skilled in the art is familiar with suitable cells to express a DNA according to the invention, which is present in an expression vector. Examples of such cells comprise the E. coli strains HB101, DH1, x1776, JM101, JM109, BL21 and SG13009, the yeast strain Saccharomyces cerevisiae and Pichia pastoris, the latter being preferred, the animal cells L, 3T3, FM3A, CHO, Vero, HeLa and COS, the latter being preferred, as well as the insect cells sf9.

The person skilled in the art also knows how to insert a DNA according to the invention in an expression vector. In addition, he knows conditions of transforming cells and transfecting cells, respectively, and then cultivating them. He is also familiar with processes of isolating and purifying the fusion polypeptide expressed by the DNA according to the invention.

A further subject matter of the present invention relates to an antibody directed against an above fusion polypeptide. Such an antibody can be prepared by common methods. It may be polyclonal and monoclonal, respectively. For its preparation it is favorable to immunize animals - particularly rabbits or chickens for a polyclonal antibody and mice for a monoclonal antibody - with an above fusion polypeptide. Further "boosters" of the animals can be effected with the same fusion polypeptide. The polyclonal antibody may then be obtained from the animal serum and egg yolk, respectively. For the preparation of the monoclonal antibody, animal spleen cells are fused with myeloma cells.

By means of the present invention it is possible to influence the interactions between proteins. This can be done by administering conjugates according to the invention and by using DNA according to the invention in a gene therapy. In particular, the unbalanced interactions can be remedied in the case of an incomplete interleukin-6 receptor. The present invention distinguishes itself in that it can be used in a cost-effective manner. This manifests itself particularly in the administration of conjugates according to the invention to influence the unbalanced interactions in the case of an incomplete interleukin-6 receptor.

Furthermore, the present invention is suited for the *ex vivo* expansion of stem cells, particularly human stem cells. In this connection, it is especially remarkable that it is

possible by means of a conjugate H-IL-6 according to the invention to obtain more stem cell colonies in the soft agar than possible with the individual components IL-6 and sIL-6R. Thus, the present invention also represents an important contribution to the well-calculated influence of blood cell formation.

By means of a fusion polypeptide H-IL-6 which comprises the sequence of fig. 3 as IL-6 polypeptide, the present invention also provides a product which is suitable as IL-6 receptor antagonist. Such a product is of great therapeutic significance.

The carrying-out of the present invention can be controlled by the antibodies according to the invention.

Brief description of the drawing

Fig. 1 shows the amino acid (DNA) sequence of a fusion polypeptide H-IL-6 according to the invention. Sequences for the restriction enzyme SalI (GTCGAC), the signal peptide (MLAVGCALLAALLAAPGAA) and the linker (RGGGGSGGGGSGGGGSVE) are indicated. The linker links the COOH terminus of human sIL-6R with the NH_2 terminus of human IL-6.

Fig. 2 shows the amino acid (DNA) sequence of a fusion polypeptide H-IL-6 according to the invention. Sequences for the restriction enzyme SalI (GTCGAC), the signal peptide (MLAVGCALLAALLAAPGAA) and the linker (RGGGGSGGGGSVE) are indicated. The linker links the COOH terminus of human sIL-6R with the NH_2 terminus of human IL-6.

Fig. 3 shows the amino acid sequence of the IL-6 polypeptide present in a fusion polypeptide H-IL-6 according to the invention.

Fig. 4 shows the expansion and colony forming capacities of a fusion polypeptide H-IL-6 according to the invention.

The invention is explained by the below examples.

Example 1: Preparation of a DNA according to the invention

The DNA of fig. 1 was prepared. For this purpose, human IL-6R cDNA (Schooltink et al., Biochem. J. (1991) 277, 659-664) was used. This cDNA was cloned into the expression plasmid pCDM8 via restriction site Xho I (Müllberg et al., Eur. J. Immunol. (1993) 23, 473-480). By means of a polymerase chain reaction (PCR), an sIL-6R fragment was generated by using the primer (1) (pCDM8 5' primer: 5' TAATACGACTCACTATAGGG3') and primer (2) (sIL-6R 3' primer: 5'CCGCTCGAGCTGGAGGACTC-CTGGA 3') under normal conditions. After being cut with restriction enzymes Hind III and Xho I, this fragment was cloned into the open plasmid pCDM8. The plasmid pCDM8-sIL-6R formed. Thereafter, a second PCR reaction was carried out with IL-6 cDNA which had also been cloned into the expression plasmid pCDM8 by using Xho I. The primers (3) (IL-6-5' primer: 5' CGGCTCGAGCCAGTACCCCCAGGAGAA3') and primer (4) (pCDM8 3' primer: 5'CCACAGAAGTAAGGTTCCTT3') were used. The PCR product was cut with restriction enzymes Xho I and Not I and cloned into plasmid pCDM8-sIL-6R. The plasmid pCDM8-sIL-6R-IL-6 formed. Thereafter, a synthetic linker was prepared which consisted of two oligonucleotides: primer (5) (5'TCGAGGAGGTGGAGGTTCTGGAGGTGGAGGTTCTGGAGGTGGAGGTTCTG3') and primer (6) (5'TCGACAGAACCTCCACCTCCAGAACCTCCACCTCCAGAACCTCCA-CCTCC3'). Oligonucleotides (5) and (6) were combined according to standard methods into a double strand and then cloned into the plasmid pCDM8-sIL-R-IL-6 digested by the restriction enzyme Xho I. The plasmid pCDM8-H-IL-6 formed.

Example 2: Preparation of a DNA according to the invention

The DNA of fig. 2 was prepared. For this purpose, the steps as described in Example 1 were carried out. However, the following primers were used as primers (5) and (6): primer (5) (5'TCGAGGACGTGGAGGTTCTGGAGGTGGAGGTTCTG3') and primer (6) (5'TCGACAGAACCTCCACCTCCAGAACCTCCACCTCC3'). The plasmid pCDM8-H-IL-6-(2) was obtained.

Example 3: Expression of a fusion polypeptide according to the invention

COS-7 cells were transfected with pCDM8-H-IL-6 of Example 1 and pCDM8-H-IL-6(2) of Example 2, respectively, by means of electroporation. 10^7 COS-7 cells were electroporated with 20 μg plasmid by means of a gene pulser (Bio-Rad) at 960 μF and 230 V. 48 h after the transfection, the cells were radioactively labeled metabolically using [^{35}S] cysteine/methionine for 4 h and incubated with amino acids which were not labeled radioactively for 2 h. The supernatant from cell lysate and cell supernatant was immunoprecipitated according to standard methods (Müllberg et al., Eur. J. Immunol. (1993) 23, 473-480) using an anti-IL-6 antibody and made visible by autoradiography after SDS gel electrophoresis. Transfected COS-7 cells secreted a 70-75 kDa protein which was recognized by an anti-IL-6 antibody and was not formed by non-transfected cells.

Supernatants of transfected COS-7 cells were separated by SDS gel electrophoresis, transferred to nitrocellulose and detected with an anti-IL-6 antibody. Again, transfected COS-7 cells expressed a 70-75 kDa protein which was recognized by an anti-IL-6 antibody.

Supernatants of transfected COS-7 cells were investigated by means of a commercial ELISA for IL-6 (CLB, Amsterdam, Netherlands) and sIL-6R (Seromed, Gießen, FRG). H-IL-6 was

detected by means of both ELISAs. The concentration of H-IL-6 in the cell supernatant was about 1 µg/ml.

Example 4: Stimulation of the haptoglobin expression by a fusion polypeptide according to the invention

The human hepatoma cell lines HepG2, HepG2-IL-6 and HepG2-PDI were used.

HepG2 cells (ATCC HB 8065) are stimulated to express haptoglobin by IL-6, but not by sIL-6R.

HepG2-IL-6 cells were obtained by stable transfection of HepG2 cells with a human IL-6 expression plasmid. On account of the IL-6 expression these cells down-regulate endogenous IL-6R and thus express no IL-6R. HepG2-IL-6 cells are not stimulated to express haptoglobin by IL-6, but by sIL-6R.

HepG2-PDI cells were obtained by stable transfection of HepG2 cells with a human IL-6 expression plasmid. For this purpose, the expression plasmid included an IL-6 cDNA by which the expressed IL-6 protein included a COOH-terminal retention signal for the endoplasmic reticulum (ER). As a result, these cells did not only retain the expressed IL-6 but also IL-6R in the ER. In contrast to HepG2-IL-6 cells, HepG2-PDI cells do not secrete IL-6 and can only be stimulated to express haptoglobin by a combination of IL-6 and sIL-6R.

The above hepatoma cell lines were cultivated under standard conditions in 96-well cell culture plates (Rose-John et al., J. Biol. Chem. 268 (1993), 22084-22091). The cells were stimulated with IL-6, sIL-6R, IL-6 + sIL-6R and cell supernatants, respectively, which originated from COS-7 cells of Example 3, transfected with pCDM8-H-IL-6, pCDM8-H-IL-6(2) and pCDM8, respectively, for 18 h. The cell supernatant was collected and the haptoglobin concentration

in the supernatant was determined by means of ELISA (cf. Table 1).

Table 1

Stimulation of the haptoglobin expression

	IL-6	sIL-6R	IL-6 + sIL-6R	H-IL-6	control
HepG2	+	-	+ +	+ + +	-
HepG2-IL-6	-	+ +	+ +	+ + +	-
HepG2-PDI	-	-	+ +	+ + +	-

It showed that a fusion polypeptide according to the invention, H-IL-6, is capable of stimulating the expression of haptoglobin in cells, i.e. of influencing the interactions between proteins.

Example 5: Expansion and colony formation of human CD34$^+$ cells by a fusion polypeptide according to the invention

Cells which express the surface marker CD34 were isolated from human bone marrow and blood of patients whose stem cells had been mobilized by injection of G-CSF, respectively. 6000 of these cells were plated in 3 ml medium in cell culture vessels. After two weeks it turned out that an incubation of the cells with cytokines SCF, IL-3 and H-IL-6 (fusion polypeptide according to the invention) as well as SCF, IL-3 and IL-6 caused strong proliferation. 1000 cells of the resulting cells were plated into new cell culture vessels. After two weeks in a standardized colony induction experiment, the cells treated with SCF, IL-3 and H-IL-6 were capable of forming about three times more colonies than cells treated with SCF, IL-3 and IL-6.

This result shows that cells stimulated by a fusion polypeptide H-IL-6 according to the invention have a greater

colony-forming potential than cells stimulated by Il-6 (cf. fig. 4).